

Microbial Desulfurization of Dibenzothiophene

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Abstract

Concerning the sulfur removal from coal before combustion there is considerable interest in microbial methods as pyrite oxidation and elimination of organically bound sulfur from coal. Using organic sulfur compounds relevant for coal the mechanism of desulfurization was investigated. We isolated a defined mixed culture (FODO) capable to utilize dibenzothiophene as sole sulfur source for growth, while benzoate was used as carbon source. The mixed culture FODO consists of an *Alcaligenes denitrificans* subspecies and a *Brevibacterium* species. Two metabolites of the degradation and dibenzothiophene-5-dioxide. The subsequent degradation of dibenzothiophene-5-dioxide used as sole sulfur source results in a release of sulfate ions into the medium. The results suggest a sulfur specific oxidative mechanism for removal of sulfur from dibenzothiophene.

Introduction

Coal combustion causes emissions such as dust, NO_x and SO_2 , which, if not diminished, are of environmental concern. To prevent SO_2 emissions sulfur removal from coal before combustion can partly be achieved by mechanical and chemical processes (1). However, for future application microbial methods are judged to represent a possible tool for sulfur removal from coal (2;3). Coal contains, apart from pyritic sulfur, traces of sulfates and elementary sulfur, a considerable amount of organically bound sulfur. This so-called organic sulfur is an element of the chemical coal structure integrated as thiol, disulfide, sulfide or thiophene in the macromolecular coal matrix (4;5). Also the occurrence of sulfur organo metallic species is established for high sulfur coals (6). For the removal of organic sulfur by means of biotechnological methods covalent C-S bonds have to be cleaved. In consideration of the inhomogenous structure of the coal, the enzymes involved should therefore be specific for the sulfur of different sulfur bonding types (regiospecificity). Experience is still limited, concerning the metabolic pathway involved in the removal of reduced sulfur from coal relevant model compounds as dibenzothiophene (DBT). Early reports suggested, that in bacterial DBT degradation the thiophene nucleus remained untouched (9). However sulfate release by an oxidative bacterial attack on DBT is reported by Isbister and Doyle (7) and Kargi and Robinson (8).

This work contributes to the oxidative bacterial desulfurization of DBT, using DBT as sole sulfur source for bacterial growth.

Material and Methods

Chemicals

Dibenzothiophene (DBT) was purchased from Merck, Hohenbrunn, FRG; dibenzothiophene sulfone (DBT-5-dioxide), benzyl methyl sulfide, dibenzyl disulfide, dibenzyl sulfide, diphenyl sulfide and poly (phenylene sulfide) from Aldrich-Chemie, Steinheim, FRG. Dibenzothiophene-5-oxide (DBT-5-oxide) was prepared as described by Gilman and Esmay (10). The product of the synthesis was contaminated with DBT and DBT-5-dioxide. Both of which were removed by high pressure liquid chromatography (HPLC). The identity of DBT-5-oxide was confirmed by GC-MS, the purity (98.3% DBT-5-oxide contaminated with 1.7% DBT-5-dioxide) by HPLC and GC. All other chemicals were obtained at the highest purity from commercial sources.

Apparatus and Analytical Methods

DBT was quantitatively removed from bacterial culture fluid by extracting with CH_2Cl_2 using equal volumes. DBT quantification was carried out by gas chromatography (Varian, mod. 3400, Darmstadt, FRG) using a Chrompack Sil 5 capillary column (25mX0.22mm) and a flame ionization detector with the following oven temperature program: 80°C for 5 min, 10°C/min to 300°C which was held for 5 min.

For metabolite characterization, samples of culture fluid were prepared by centrifugation at 10,000 g for 15 minutes to remove particles and cells. Metabolites of DBT degradation in the supernatant were detected at 220 nm or 290 nm after high pressure liquid chromatography (HPLC) using following equipment: Liquid chromatograph 655A-11 with autosampler (Merck, Darmstadt, FRG) and variable wavelength monitor (Knauer, Bad Homburg, FRG); columns (Knauer): length 250 mm, internal diameter 4 mm or 16 mm filled with LiChrosorb RP8 particles (Merck) of 5 μm in diameter; solvent system: 50% acetonitril (v/v), 50% water (v/v) containing 0.12% H_2PO_4 (v/v).

Cochromatography was carried out with synthesized DBT-5-oxide and authentic DBT-5-dioxide at approx. the same concentrations as the metabolites.

For identification of metabolites the UV-spectra (spectrophotometer: Shimadzu UV-210 A, Langenfeld, FRG) were compared with authentic DBT-5-dioxide.

GC-MS analyses were performed with a double focussing mass spectrometer (Finnigan MAT-8200, EI 70 eV) and an INCOS datasystem. The determination of sulfate ion concentration in the culture fluid was routinely carried out by the method of Cypionka and Pfennig (11). Additionally sulfate and sulfite ion concentration are detected by ion chromatography (Dionex 2000-I). Growth of the cultures was monitored either spectrophotometrically by measuring the optical density at 578 nm with an Eppendorf photometer 1101 M (Eppendorf, Hamburg, FRG) or as protein using the Lowry method (12).

Media

Sulfur limitation required scrupulously cleaned glassware to prevent bacterial growth on contaminant sulfur. The medium consisted of a salt solution containing, per liter, 2.19 g NH_4Cl , 0.27 g KH_2PO_4 , 0.2 g $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$, 0.1 g $\text{Ca}(\text{NO}_3)_2 \times 4 \text{ H}_2\text{O}$, 10 mg $\text{FeCl}_3 \times 6 \text{ H}_2\text{O}$ and 1 ml of trace element solution. The medium was adjusted with 20 mM tris/HCl to pH 7.4 and was supplemented with 10 mM benzoate. The required amount of organic sulfur sources were put into culture tubes or flasks from stock solutions (DBT dissolved in diethyl ether, DBT-5-oxide/DBT-5-dioxide dissolved in CH_2Cl_2). After the organic solvents had been evaporated medium was added. The tightly closed culture tubes and flasks were sterilized at 121°C for 20 minutes. All organic sulfur sources used are stable as proved by GC and HPLC.

Enrichment and Isolation of Organisms

Enrichment of suitable microorganisms was achieved in a pneumatically operated percolator with external loop as described by Codner (13) containing 33% (w/w) sterilized postbog, 33% (w/w) raschig-rings and 33% (w/w) municipal sewage sludge as inoculum. To adapt the microbial population selectively to organic sulfur compounds, the percolator was supplemented with dibenzothiophene, benzyl methyl sulfide, dibenzyl disulfide, dibenzyl sulfide, diphenyl sulfide and poly(phenylene sulfide) at concentrations of the percolator fluid were incubated at 30°C on a rotary shaker at 170 rpm in 10 ml screw-cap culture tubes (3 ml medium) with DBT (200 µM) as sole sulfur source and glucose (10 mM), glycerol (10 mM) or benzoate (10 mM) as carbon source. The isolation of organisms was carried out as described by Cook and Hutter (14), the vitamin solution being omitted. Bacterial isolates were characterized according to their gram reaction, fatty acid composition and physiological parameters in part by DSM (Braunschweig, FRG).

2.5 Isolation of Metabolites

Cells were removed from the culture fluid by centrifugation (10,000 g, 15 min) and the supernatant was acidified to a final concentration of 0.1 M HCl and extracted with CH₂Cl₂ (1:1 v/v). After concentrating the organic phase to approximately 1% of the original volume the preparation was used for GC-MS analyses. Samples for isolation of metabolites by HPLC were transferred to the aqueous phase again.

Results and Discussion

Isolation and Characterization of Organisms

After simultaneous adaption of an enrichment culture to several organic sulfur compounds using a percolation equipment one stable bacterial mixed culture was obtained from percolator liquid. This culture was able to grow with DBT as sole sulfur source and benzoate as sole carbon source. Without sulfur source bacterial growth was strongly limited. Analogous enrichment procedures with glucose or glycerol as carbon source were not successful. After numerous isolation steps, a two species community (FODO) was obtained. The two organisms could be distinguished by the morphology of their colonies. On nutrient broth (NB) agar plates growth of organism FO (feeding organism) was always superior compared to organism DO (degrading organism). Pure cultures of strain DO on NB agar plates formed very small and transparent colonies (diameter 0.5 - 1 mm, 30°C, 3 d), whereas colonies growing together with strain FO on the same plate are lightly yellow with a diameter of 4-5 mm (30°C, 3 d). This effect could be due to the need of diffusable nutrients or vitamins by strain DO.

Pure cultures of each strain were not able to grow with DBT as sole sulfur source.

Strain FO was identified as a non-motile subspecies of *Alcaligenes denitrificans* and strain DO as a non-motile *Brevibacterium* species.

Physiology of growth

The mixed culture FODO cultured with DBT (200 µM) as sole sulfur source and benzoate as carbon source grew with a doubling time of 8.5 h at the pH optimum of 7.4.

A molar growth yield of 9.8 kg protein per mol of sulfur was obtained with low concentrations (1-30 µM) of DBT or sulfate as sulfur source (Fig. 1). This indicates that the DBT-sulfur was as accessible to the culture as sulfate.

Comparable ranges of molar growth yields are described by Cook and Hutter (14) for bacteria, isolated from sewage and soil.

Table 1 showed that bacterial growth and DBT-degradation remained almost constant, in spite of sulfate in varying concentrations (0-100 μM) was present as a second sulfur source. This indicates that at least the first step in DBT degradation is not repressed by sulfate.

Catabolism of DBT

The bacterial mixed culture FODO was grown on DBT (200 μM) with benzoate as carbon source. In the course of bacterial growth DBT was degraded to a final concentration of 71 μM concomitant to growth. In parallel sterile controls the DBT concentration remained unchanged.

Furthermore the formation of two metabolites of DBT degradation was observed (Fig. 2). The metabolite occurring first was identified by GC-MS and cochromatography (HPLC) as DBT-5-oxide. The second metabolite was identified by cochromatography (HPLC) and UV-spectrometry as DBT-5-dioxide (Fig. 3 and Tab. 2). DBT-5-oxide was produced concomitant to bacterial growth up to a final concentration of 42 μM after 80 h incubation. At the end of the logarithmic growth phase production of DBT-5-dioxide was quantified as a final concentration of 18 μM (Fig. 2).

The yield of the products obtained, was about 46% of the amount which would be achieved by totally conversion of DBT to the concentration of about 15 μM sulfur is necessary for an optimal growth of the cells. Several organisms are able to accumulate sulfur intracellularly (15) but this seems not to be true with the mixed culture FODO because DBT grown cells, washed and incubated in sulfate free medium are strongly limited in growth due to sulfur depletion.

The discrepancy in sulfur balance implies that at least one other sulfur compound has to be accumulated during growth. However no sulfate, sulfite or other additional metabolite could be identified up to now.

In order to prove whether DBT-5-oxide and DBT-5-dioxide are utilized by the bacterial culture as sulfur sources too, growth experiments using this compound (25 μM) as sole sulfur source with benzoate as carbon source were carried out. As shown in Fig. 4 both the sulfoxide and the sulfone were degraded in the course of cell growth, whereas the growth of control cultures without any sulfur source was negligible.

Using high concentrations of DBT-5-dioxide (2 mM) as sole source of sulfur with benzoate as carbon source, in the

stationary phase of growth 21.2% (424 μM) of DBT-5-dioxide was degraded and a concentration of 200 μM sulfate, but no sulfite was detected in the supernatant culture fluid. In sterile controls neither sulfate nor sulfite could be measured. Considering the amount of 15 μM sulfur the bacterial cultures needed for optimal growth, these results confirm the aforementioned hypothesis, that at least one additional unknown sulfur compound accumulates.

Further investigations are directed to isolate this "missing sulfur compound" and the first sulfur free metabolite to examine the C-S bond cleavage occurring in the desulfurization of DBT.

The presented data indicate a sulfur specific attack on DBT by a bacterial two species community which utilizes DBT as sole source of sulfur. We propose that sulfur elimination proceeds via the corresponding sulfoxide and sulfone (Fig. 5).

This is the first report of the bacterial degradation of DBT serving as sulfur source, though similar reactions have been reported: The microbial oxidation of thiophenes to the corresponding sulfoxides and sulfones is reported by Holland et al (16) and Fedorak et al (17), but no further degradation could be achieved. Kaufmann and Kearney (18) reported on the degradation of a methylthio-S-triazine by soil bacteria. The desulfurization of this organic sulfide proceeds through the sulfoxide and the sulfone whereby methyl sulfonic acid is suspected to be an intermediate (14). A *Pseudomonas* species is described to desulfurize DBT by cooxidation with benzoate by Isbister and Doyle (7). Using DBT as carbon and energy source for growth of *Sulfolobus acidocaldarius* sulfate release was measured by Kargi and Robinson (8). However biochemical reactions of the aforementioned DBT degradation are not reported in detail, the initial attack may be of similar type as presented in this work. This initial reaction occurring in DBT degradation (Fig. 5) support the approach to develop specific mechanisms for microbial removal of organically bound sulfur from coal.

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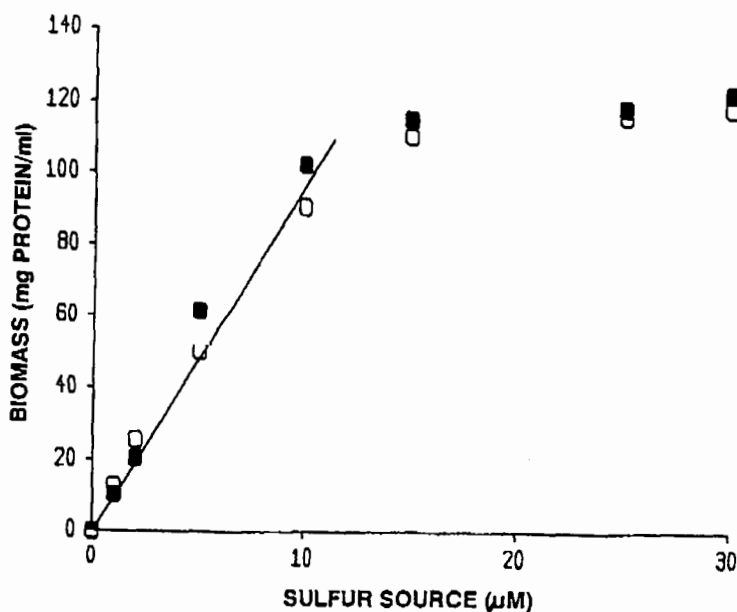


Figure 1: Biomass production at the stationary growth phase of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. at different concentrations of sulfur sources. □ dibenzothiophene, ■ sulfate

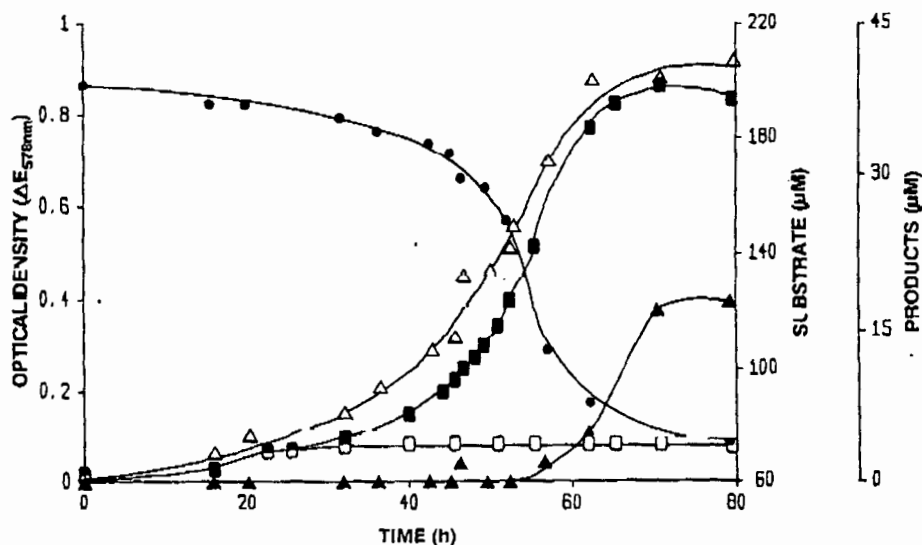


Figure 2: Growth of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with dibenzothiophene as sole sulfur source and benzoate as carbon source. ■ optical density, □ optical density without sulfur source added, ● dibenzothiophene, △ dibenzothiophene-5-oxide, ▲ dibenzothiophene-5-dioxide.

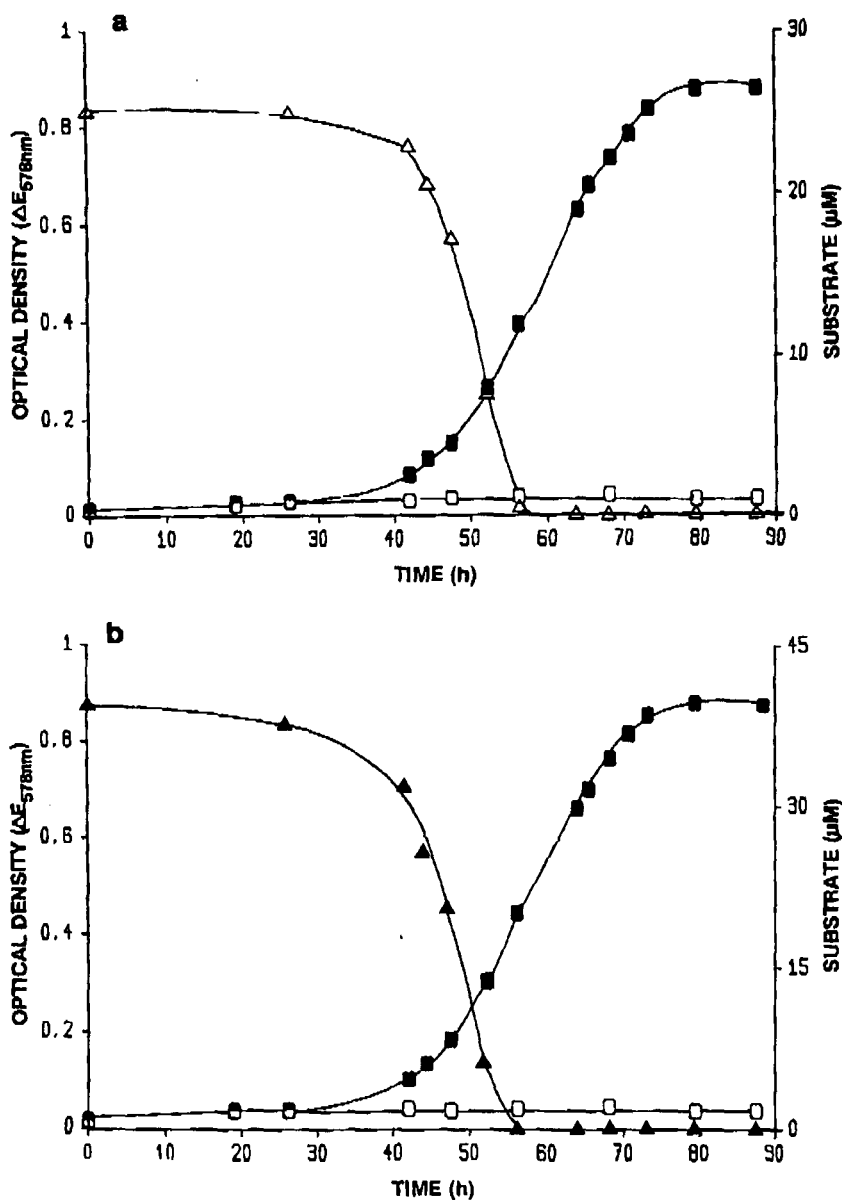


Figure 4: Growth of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with dibenzothiophene-5-oxide (a) and dibenzothiophene-5-dioxide (b) as sole sulfur source and benzoate as carbon source. ■ optical density, □ optical density without sulfur source added, △ dibenzothiophene-5-oxide, ▲ dibenzothiophene-5-dioxide.

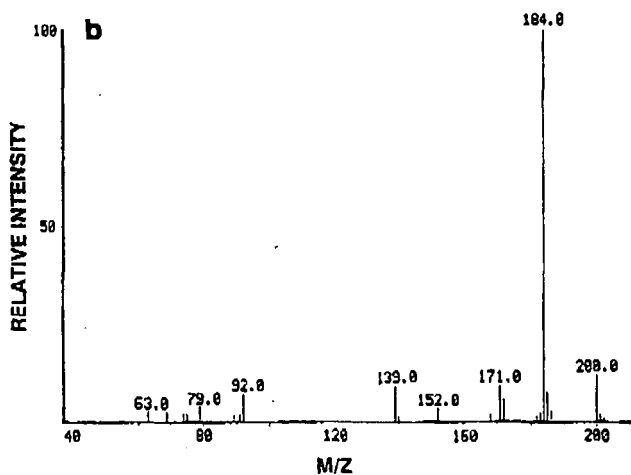
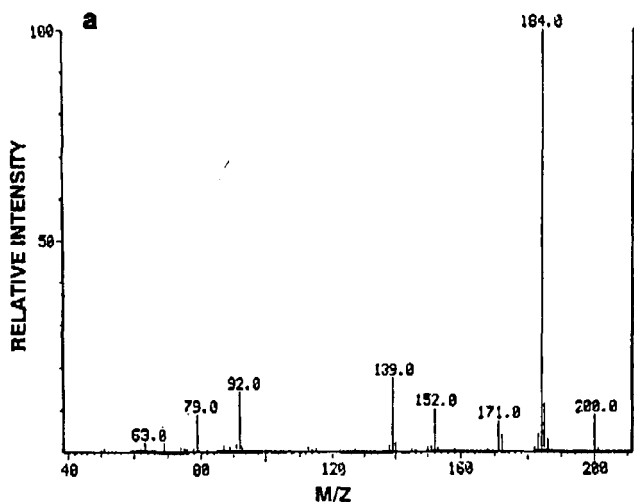


Figure 3: Mass spectra of (a) dibenzothiohene-5-oxide produced from dibenzothiohene by the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. and (b) authentic dibenzothiohene-5-oxide (library search).

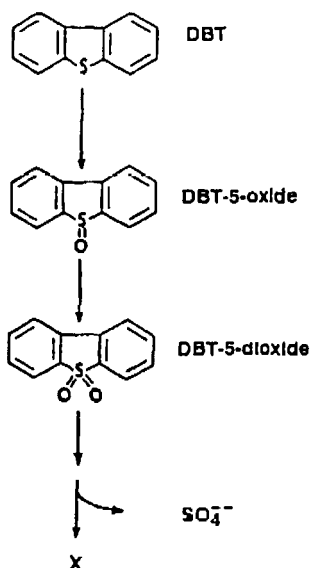


Figure 5:
Proposed initial reactions of the desulfurization of dibenzothiophene (DBT) by the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp..

Table 1: Utilization of dibenzothiophene (100 μM) by the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with benzoate as carbon source in presence of various amounts of sulfate.

Sulfate (μM)	optical density ($E_{578\text{nm}}$)	dibenzothiophene degradation (%)
0	0.96	85.5
2	1.01	84.5
5	1.02	80.9
10	0.92	90.0
15	1.01	79.5
30	0.96	84.5
50	0.99	91.8
100	1.00	89.6

Table 2: Identification of metabolites from dibenzothiophene (DBT) released during growth of mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp.

Product	Cochromatography* retention time (min)	UV-spectrometry** maxima (nm)
DBT-5-oxide	5.1	not assayed
DBT-5-dioxide	7.1	236/244/280/292/322

* HPLC, retention times are identical with authentic chemicals

** Maxima are identical with authentic DBT-5-dioxide